

**TEST METHOD PROTOCOL
for the BALB/c 3T3 Neutral Red Uptake Cytotoxicity Test**

**A Test for Basal Cytotoxicity for an *In Vitro* Validation Study
Phase Ib**

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Prepared by

**The National Toxicology Program (NTP) Interagency Center for the Evaluation of
Alternative Toxicological Methods (NICEATM)**

**Based on Standard Operating Procedure Recommendations from an
International Workshop Organized by the Interagency Coordinating Committee
on the Validation of Alternative Methods (ICCVAM)**

**National Institute of Environmental Health Sciences (NIEHS)
National Institutes of Health (NIH)
U.S. Public Health Service
Department of Health and Human Services**

TEST METHOD PROTOCOL

The BALB/c 3T3 Neutral Red Uptake Cytotoxicity Test A Test for Basal Cytotoxicity Phase Ib

I. PURPOSE

The purpose of this study is to evaluate the cytotoxicity of test chemicals using the BALB/c 3T3 Neutral Red Uptake (NRU) cytotoxicity test. The data will be used to evaluate the intra- and inter-laboratory reproducibility of the assay and effectiveness of the cytotoxicity assay to predict the starting doses for rodent acute oral systemic toxicity assays. This test method protocol outlines the procedures for performing the cytotoxicity test and supports the *in vitro* validation study organized by NICEATM and the European Centre for the Validation of Alternative Methods (ECVAM) and sponsored by NIEHS, U.S. Environmental Protection Agency, and ECVAM. This test method protocol applies to all personnel involved with performing the cytotoxicity assay.

A. BALB/c 3T3 Neutral Red Uptake Cytotoxicity Test

The 3T3 NRU test will be performed to analyze the *in vitro* toxicity of three (3) blinded/coded test chemicals. This test will be used to determine IC₂₀, IC₅₀, and IC₈₀ values for the predetermined set of test chemicals of varying toxicities.

II. SPONSOR

- A. Name: National Institute of Environmental Health Sciences (NIEHS); The National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM)
- B. Address: P.O. Box 12233
Research Triangle Park, NC 27709
- C. Representative: *Named Representative*

III. IDENTIFICATION OF TEST AND CONTROL SUBSTANCES

- A. Test Chemicals: *Blinded Chemicals (3)*
- B. Controls:
- | | |
|----------------------|--|
| Positive: | Sodium Lauryl Sulfate |
| Vehicle (Negative): | Assay medium (DMEM containing 5% NBCS, 4 mM L-Glutamine, 100 IU/mL Penicillin, 100 µg/mL Streptomycin) |
| Solvent (as needed): | Assay medium with appropriate solvent used to prepare the test chemicals (Section VII.E) |

IV. TESTING FACILITY AND KEY PERSONNEL

- 1) Name:

- 2) Address:
- 3) Study Director:
- 4) Laboratory Technician(s):
- 5) Scientific Advisor:
- 6) Quality Assurance Director:
- 7) Safety Manager:
- 8) Facility Management:

A. Test Schedule

- 1) Proposed Experimental Initiation Date:
- 2) Proposed Experimental Completion Date:
- 3) Proposed Report Date:

V. TEST SYSTEM

The NRU cytotoxicity assay procedure is a cell survival/viability chemosensitivity assay based on the ability of viable cells to incorporate and bind neutral red (NR), a supravital dye. NR is a weak cationic dye that readily penetrates cell membranes by non-ionic diffusion and accumulates intracellularly in lysosomes. Alterations of the cell surface or the sensitive lysosomal membrane lead to lysosomal fragility and other changes that gradually become irreversible. Such changes brought about by the action of xenobiotics result in a decreased uptake and binding of NR. It is thus possible to distinguish between viable, damaged, or dead cells, which is the basis of this assay.

Healthy mammalian cells, when maintained in culture, continuously divide and multiply over time. A toxic chemical, regardless of site or mechanism of action, will interfere with this process and result in a reduction of the growth rate as reflected by cell number. Cytotoxicity is expressed as a concentration dependent reduction of the uptake of the NR after chemical exposure thus providing a sensitive, integrated signal of both cell integrity and growth inhibition.

VI. DEFINITIONS

- A. Hill function:** a four parameter logistic mathematical model relating the concentration of test chemical to the response being measured in a sigmoidal shape.

$$Y = \text{Bottom} + \frac{\text{Top} - \text{Bottom}}{1 + 10^{(\log \text{IC}_{50} - X) \text{HillSlope}}}$$

where Y= response, X is the logarithm of dose (or concentration), Bottom is the minimum response, Top is the maximum response, logIC₅₀ is logarithm of X at the response midway between Top and Bottom, and HillSlope describes the steepness of the curve.

- B. Documentation:** all methods and procedures will be noted in a Study Workbook; logs will be maintained for general laboratory procedures and equipment (e.g., media preparation, test chemical preparation, incubator function); all optical density data obtained from the spectrophotometer plate reader will be saved in electronic and paper formats; all calculations of IC_x values and other derived data will be in electronic and paper format; all data will be archived.

VII. PROCEDURES

A. Materials

1. Cell Lines

BALB/c 3T3 cells, clone 31

CCL-163, LGC Reference Materials, Customer Service, Queens Road, Teddington, Middlesex, TW110LY, UK

CCL-163, American Type Culture Collection [ATCC], Manassas, VA, USA)

2. Technical Equipment

[Note: Suggested brand names/vendors are listed in parentheses. Equivalents may be used.]

- 1) Incubator: 37°C ± 1°C, 90 % ± 5 % humidity, 5.0 % ± 1 % CO₂/air
- 2) Laminar flow clean bench/cabinet (standard: "biological hazard")
- 3) Water bath: 37°C ± 1°C
- 4) Inverse phase contrast microscope
- 5) Sterile glass tubes with caps (e.g., 5 mL)
- 6) Centrifuge (optionally: equipped with microtiter plate rotor)
- 7) Laboratory balance
- 8) 96-well plate spectrophotometer (i.e., plate reader) equipped with 540 nm ± 10 nm filter
- 9) Shaker for microtiter plates
- 10) Cell counter or hemocytometer
- 11) Pipetting aid
- 12) Pipettes, pipettors (multi-channel and single channel), dilution block
- 13) Cryotubes
- 14) Tissue culture flasks (e.g., 75 - 80 cm², 25 cm²)
- 15) 96-well flat bottom tissue culture microtiter plates (e.g., Nunc # 167 008; Falcon tissue culture-treated)
- 16) pH paper (wide and narrow range)

[Note: Tissue culture flasks and microtiter plates should be prescreened to ensure that they adequately support the growth of 3T3 cells.]

3. Chemicals, Media, and Sera

- Dulbecco's Modification of Eagle's Medium (DMEM) without L-Glutamine; should have high glucose [4.5gm/l] (e.g., ICN-Flow Cat. No. 12-332-54)
- L-Glutamine 200 mM (e.g., ICN-Flow # 16-801-49)

- New Born Calf Serum (NBCS or NCS) (e.g., Biochrom # SO 125)
- 0.05 % Trypsin/0.02 % EDTA solution (e.g., SIGMA T 3924, ICN-Flow, # 16891-49)
- Phosphate buffered saline (PBS) without Ca^{2+} and Mg^{2+} (for trypsinization)
- Hanks' Balanced Salt Solution (HBSS) without Ca^{2+} and Mg^{2+} (CMF-HBSS)
- Dulbecco's Phosphate Buffered Saline (D-PBS) [formulation containing calcium and magnesium cations; glucose optional] (for rinsing)
- Penicillin/streptomycin solution (e.g. ICN-Flow # 16-700-49)
- Neutral Red (NR) Dye – tissue culture-grade; liquid form (e.g., SIGMA N 2889); powder form (e.g., SIGMA N 4638)
- Dimethyl sulfoxide (DMSO), U.S.P. analytical grade (Store under nitrogen @ -20°C)
- Ethanol (ETOH), U.S.P. analytical grade (100 %, non-denatured for test chemical preparation; 95 % can be used for the desorb solution)
- Glacial acetic acid, analytical grade
- Distilled H_2O or any purified water suitable for cell culture (sterile)
- Sterile paper towels (for blotting 96-well plates)

[Note: Due to lot variability of NBCS/NCS, first check a lot for growth stimulating properties with 3T3 cells (approximately 20-24 h doubling time) and then reserve a sufficient amount of NBCS/NCS. May use pre-tested serum lot from Phase Ia of the validation study if the serum has been stored under appropriate conditions and shelf-life has not expired.]

B. Preparations of Media and Solutions

[Note: All solutions (except NR stock solution, NR medium and NR desorb), glassware, pipettes, etc., shall be sterile and all procedures should be carried out under aseptic conditions and in the sterile environment of a laminar flow cabinet (biological hazard standard). All methods and procedures will be adequately documented.]

1. Media

DMEM (buffered with sodium bicarbonate) supplemented with (final concentrations in DMEM are quoted):

- for freezing (Freeze Medium); contains 2X concentration of NBCS/NCS and DMSO of final freezing solution

40 %	NBCS/NCS
20 %	DMSO
- for routine culture (Routine Culture Medium)

10 %	NBCS/NCS
4 mM	Glutamine
- for solubility testing and test chemical dilution (Chemical Dilution Medium)

4 mM	Glutamine
200 IU/mL	Penicillin
200 µg/mL	Streptomycin
- for dilution of NR stock solution (NR Dilution Medium)

5 %	NBCS/NCS
4 mM	Glutamine
100 IU/mL	Penicillin
100 µg/mL	Streptomycin

[Note: The Chemical Dilution Medium with test chemical will dilute the serum concentration of the Routine Culture Medium in the test plate to 5 %. Serum proteins may mask the toxicity of the test substance, but serum cannot be totally excluded because cell growth is markedly reduced in its absence.]

Completed media formulations should be kept at approximately 2-8° C and stored for no longer than two weeks.

2. Neutral Red (NR) Stock Solution

The liquid tissue culture-grade stock NR Solution will be the first choice for performing the assay (e.g., SIGMA #N2889, 3.3 mg/mL). Store liquid tissue culture-grade NR Stock Solution at the storage conditions and shelf-life period recommended by the manufacturer.

If the liquid form is not available, the following formulation can be prepared.

EXAMPLE: 0.33 g NR Dye powder in 100 mL H₂O

The NR Stock Solution (powder in water) should be stored in the dark at room temperature for up to two months.

3. Neutral Red (NR) Medium

EXAMPLE:

1 mL (3.3 mg NR dye/mL)	NR Stock Solution
99 mL	NR Dilution Medium (pre-warmed to 37° C)

The final concentration of the NR Medium is 33 µg NR dye/mL.

[Note: The NR medium may be centrifuged at approximately 600 x g for 10 min (to remove NR crystals). The NR Medium shall be filtered (e.g., Millipore filtering, 0.2 – 0.45 µm pore size) to reduce NR crystals. The temperature of the NR Medium should be maintained at 37° C (e.g., in a waterbath) before adding to the cells and will be used within 15 minutes after removing from 37° C storage. Aliquots of NR Medium can be made on the day of testing and maintained at 37° C for later use.]

4. Ethanol/Acetic Acid Solution (NR Desorb)

1 %	Glacial acetic acid solution
50 %	Ethanol
49 %	H ₂ O

C. Methods

1. Cell Maintenance and Culture Procedures

BALB/c 3T3 cells are routinely grown as a monolayer in tissue culture grade flasks (e.g., 75 - 80 cm²) at 37°C ± 1°C, 90 % ± 5 % humidity, and 5.0 % ± 1 % CO₂/air. The cells should be examined on a daily (i.e., on workdays) basis under a phase contrast microscope, and any changes in morphology or their adhesive properties noted in a Study Workbook.

2. Receipt of Cryopreserved BALB/c 3T3 Cells

Upon receipt of cryopreserved BALB/c 3T3 cells, the vial(s) of cells shall be stored in a liquid nitrogen freezer until needed.

3. Thawing Cells

Thaw cells by putting ampules into a water bath at 37°C ± 1°C. Leave for as brief a time as possible.

- Resuspend the cells in pre-warmed Routine Culture Medium and transfer into pre-warmed Routine Culture Medium in a tissue-culture flask.
- Incubate at 37°C ± 1°C, 90 % ± 5 % humidity, and 5.0 % ± 1 % CO₂/air.
- When the cells have attached to the bottom of the flask (within 4 to 24 h), decant the supernatant and replace with fresh pre-warmed (37°C) medium. Culture as described above.
- Passage at least two times before using the cells in a cytotoxicity test.

A fresh batch of frozen cells from the stock lot of cells should be thawed out and cultured approximately every two months. This period resembles a sequence of about 18 passages.

4. Routine Culture of BALB/C 3T3 Cells

When cells exceed 50 % confluence (but less than 80 % confluent) they should be removed from the flask by trypsinization:

- Decant medium, rinse cultures with 5 mL PBS or Hanks' BSS (without Ca²⁺, Mg²⁺) per 25 cm² flask (15 mL per 75 cm² flask). Wash cells by gentle agitation to remove any remaining serum that might inhibit the action of the trypsin.
- Discard the washing solution.
- Add 1-2 mL trypsin-EDTA solution per 25 cm² to the monolayer for a few seconds (e.g., 15-30 seconds).
- Remove excess trypsin-EDTA solution and incubate the cells at room temperature.
- After 2-3 minutes (min), lightly tap the flask to detach the cells into a single cell suspension.

5. Cell Counting

After detaching the cells, add 0.1-0.2 mL of pre-warmed (37°C) Routine Culture Medium/cm² to the flask (e.g., 2.5 mL for a 25 cm² flask). Disperse the monolayer by gentle trituration. It is important to obtain a single cell suspension for exact counting. Count a

sample of the cell suspension obtained using a hemocytometer or cell counter (e.g., Coulter counter).

6. Subculture of Cells

After determination of cell number, the culture can be sub-cultured into other flasks or seeded into 96-well microtiter plates. BALB/c 3T3 cells are routinely passaged at suggested cell densities as listed in the table (approximate doubling time is 20-24 h). The individual laboratories will need to determine and adjust the final density to achieve appropriate growth.

Table 1. Cell Densities for Subculturing

Days in Culture	Seeding Density (cells/cm ²)	Total Cells per 25 cm ² flask	Total Cells per 75 cm ² flask
2	16800	4.2×10^5	1.26×10^6
3	8400	2.1×10^5	6.3×10^5
4	4200	1.05×10^5	3.15×10^5

[Note: It is important that cells have overcome the lag growth phase when they are used for the test.]

7. Freezing Cells (procedure required only if current stock of cells is depleted)

Stocks of BALB/c 3T3 cells can be stored in sterile, freezing tubes in a liquid nitrogen freezer. DMSO is used as a cryoprotective agent.

- Centrifuge trypsinized cells at approximately 200 x g.
- Suspend the cells in cold Routine Culture Medium (half the final freezing volume) so a final concentration of $1-5 \times 10^6$ cells/mL can be attained.
- Slowly add cold Freeze Medium to the cells so that the solvent will equilibrate across the cell membranes. Bring the cell suspension to the final freezing volume. The final cell suspension will be 10 % DMSO. Aliquot the cell suspension into freezing tubes and fill to 1.8 mL.
- Place the tubes into an insulated container (e.g., styrofoam trays) and place in a freezer (-70 to -80°C) for 24 h. This gives a freezing rate of approximately 1°C/min. The laboratory needs to ensure that the freezing protocol is applicable to the 3T3 cells and that the cells are viable when removed from cryopreservation.
- Place the frozen tubes into liquid nitrogen for storage.

8. Preparation of Cells for Assays

- Cultured cells that are going to be used in seeding the 96-well plates should be fed fresh medium the day before subculturing to the plates. On the day of plate seeding, prepare a cell suspension of 2.5×10^4 cells/mL in Routine Culture Medium. Using a multi-channel pipette, dispense 100 µl Routine Culture Medium only into the peripheral wells (blanks) of a 96-well tissue culture microtiter plate (See **Section VII.F.1**). In the remaining wells, dispense 100 µl of a cell suspension of 2.5×10^4 cells/mL ($= 2.5 \times 10^3$ cells/well). The seeding density should be noted to ensure that the cells in the control wells are not

- overgrown after three days (i.e., 24 h incubation in step **b** and 48 h exposure to test chemicals). Prepare one plate per chemical to be tested.
- e) Incubate cells for 24 ± 1 h ($37^{\circ}\text{C} \pm 1^{\circ}\text{C}$, $90\% \pm 5\%$ humidity, $5.0\% \pm 1\%$ CO_2/air) so that cells form a less than half confluent monolayer. This incubation period assures cell recovery and adherence and progression to exponential growth phase.
 - e) Examine each plate under a phase contrast microscope to assure that cell growth is relatively even across the microtiter plate. This check is performed to identify experimental and systemic cell seeding errors. Record observations in the Study Workbook.

9. Determination of Doubling Time

- a) A cell doubling time procedure was performed on the initial lot of cells that was used in the first cell culture assays of Phase Ia of the Validation Study. The doubling time only needs to be determined in Phase Ib if there is a change in the lot of cells used. Establish cells in culture and trypsinize cells as per **Section VII.C.4** for subculture. Resuspend cells in NR Dilution Medium (5 % NBCS/NCS). Seed cells at 4200 cells/cm².
- b) Seed five sets of cell culture vessels in triplicate for each cell type (e.g., 15 tissue culture dishes [60mm x 15mm]). Use appropriate volume of culture medium for the culture vessels. Note number of cells placed into each culture dish. Place dishes into the incubators ($37^{\circ}\text{C} \pm 1^{\circ}\text{C}$, $90\% \pm 5\%$ humidity, $5.0\% \pm 1\%$ CO_2/air).
- c) After 4 - 6 hours (use the same initial measurement time for each subsequent doubling time experiment), remove three culture dishes and trypsinize cells. Count cells using a cell counter or hemocytometer. Cell viability may be determined by dye exclusion (e.g., Trypan Blue) if Study Director sees a need. Use appropriate size exclusion limits if using a Coulter counter. Determine the total number of cells and document. Repeat sampling at 24 h, 48 h, 72 h, and 96 h post inoculation. Change culture medium at 72 h or sooner in remaining dishes if indicated by pH drop.
- d) Plot cell concentration (per mL of medium) on a log scale against time on a linear scale. Determine lag time and population doubling time. Additional dishes and time are needed if the entire growth curve is to be determined (lag phase, log phase, plateau phase).

D. Solubility Test

The preference of solvent for dissolving test chemicals is medium, DMSO, and then ethanol. Solubility shall be determined in a step-wise procedure that involves attempting to dissolve a test chemical at a relatively high concentration with the sequence of mechanical procedures specified in **Section VII.D.2.a**. If the chemical does not dissolve, the volume of solvent is increased so as to decrease the concentration by a factor of 10, and then the sequence of mechanical procedures in **Section VII.D.2.a** are repeated in an attempt to solubilize the chemical at the lower concentrations. For testing solubility in medium, the starting concentration is 20,000 µg/ml (i.e., 20 mg/mL) in Tier 1, but for DMSO and ethanol the starting concentration is 200,000 µg/ml (i.e., 200 mg/mL) in Tier 2. Weighing out chemical for each solvent (i.e., medium, DMSO, ethanol) can be done all at once, if convenient, but solubility testing (at each tier that calls for more than one solvent) is designed to be sequential - medium, then DMSO, then ethanol – in accordance with the solvent hierarchy (see **Figure 1**). This allows for testing to stop, rather than continue testing with less preferred solvents, if the test chemical dissolves in a more preferred solvent. For example, if a chemical is soluble in medium at a particular tier, testing may stop. Likewise, if a chemical is soluble in DMSO at any tier, testing need not continue with ethanol. However, since the issue of primary importance is testing the solvents and concentrations of test chemical

required by any one tier, sequential testing of solvents may be abandoned if the lab can test more efficiently in another way.

1. Determination of Solubility

- a) Tier 1 begins with testing 20 mg/mL in Chemical Dilution Medium (see **Table 2**). Approximately 10 mg (10,000 µg) of the test chemical will be weighed into a glass tube and the weight will be documented. A volume of Chemical Dilution Medium, approximately 0.5 mL, will be added to the vessel so that the concentration is 20,000 µg/mL (20 mg/mL). The solution is mixed as specified in **Section VII.D.2.a**. If complete solubility is achieved in medium, then additional solubility procedures are not needed.
- b) If the test chemical is insoluble in Chemical Dilution Medium, proceed to Tier 2 by adding enough medium, approximately 4.5 mL, to attempt to dissolve the chemical at 2 mg/mL by using the sequence of mixing procedures specified in **Section VII.D.2.a**. If the test chemical dissolves in Chemical Dilution Medium at 2 mg/mL, no further procedures are necessary. If the test chemical does NOT dissolve, weigh out approximately 100 mg test chemical in a second glass tube and add enough DMSO to make the total volume approximately 0.5 mL (for 200 mg/mL). In another glass tube, also add approximately 100 mg test chemical to enough ethanol to make the total volume approximately 0.5 mL (for 200 mg/mL). Mix both solutions as specified in **Section VII.D.2.a** in an attempt to solubilize the test chemical. If the chemical is soluble in either solvent, no additional solubility procedures are needed.
- c) If the chemical is NOT soluble in Chemical Dilution Medium, DMSO, or ethanol at Tier 2, then continue to Tier 3 in Table 2 by adding enough solvent to increase the volume of the three Tier 2 solutions by 10 and attempt to solubilize again using the sequence of mixing procedures in **Section VII.D.2.a**. If the test chemical dissolves, no additional solubility procedures are necessary. If the test chemical does NOT dissolve, continue with Tier 4 and, if necessary, Tier 5 using DMSO and ethanol. Tier 4 begins by diluting the Tier 3 samples with DMSO or ethanol to bring the total volume to 50 mL. The mixing procedures in **Section VII.D.2.a** are again followed to attempt to solubilize the chemical. Tier 5 is performed, if necessary, by weighing out another two more samples of test chemical at ~10 mg each and adding ~50 mL DMSO or ethanol for a 200 µg/mL solution, and following the mixing procedures in **Section VII.D.2.a**.

Example: If complete solubility is not achieved at 20,000 µg/mL in Chemical Dilution Medium at Tier 1 using the mixing procedures specified in **Section VII.D.2.a**, then the procedure continues to Tier 2 by diluting the solution to 5 mL and mixing again as specified in **Section VII.D.2.a**. If the chemical is not soluble in Chemical Dilution Medium, two samples of ~ 100 mg test chemical are weighed to attempt to solubilize in DMSO and ethanol at 200,000 µg/mL (i.e., 200 mg/mL). Solutions are mixed following the sequence of procedures prescribed in **Section VII.D.2.a** in an attempt to dissolve. If solubility is not achieved at Tier 2, then the solutions (Chemical Dilution Medium, DMSO, and ethanol) prepared in Tier 2 are diluted by 10 so as to test 200 µg/mL in media, and 20,000 µg/mL in DMSO and ethanol. This advances the procedure to Tier 3. Solutions are again mixed as prescribed in **Section VII.D.2.a** in an attempt to dissolve. If solubility is not achieved in Tier 3, the procedure continues to Tier 4, and to 5 if necessary (see **Figure 1** and **Table 2**).

Table 2 Determination of Solubility in Chemical Dilution Medium, DMSO, or Ethanol

TIER	1	2	3	4	5
Total Volume Chemical Dilution Medium	0.5 mL	5 mL	50 mL		
Concentration of Test Chemical (Add ~10 mg to a tube. Add enough medium to equal the first volume. Dilute to subsequent volumes if necessary.)	20,000 µg/mL (20 mg/mL)	2,000 µg/mL (2 mg/mL)	200 µg/mL (0.20 mg/mL)		
Total Volume DMSO/Ethanol		0.5 mL	5 mL	50 mL	
Concentration of Test Chemical (Add ~100 mg to a large tube. Add enough DMSO or ethanol to equal the first volume. Dilute to subsequent volumes if necessary.)		200,000 µg/mL (200 mg/mL)	20,000 µg/mL (20 mg/mL)	2,000 µg/mL (2 mg/mL)	
Total Volume DMSO/Ethanol					50 mL
Concentration of Test Chemical (Add ~10 mg to a large tube. Add enough DMSO or ethanol to equal 50 mL.)					200 µg/mL (0.2 mg/mL)
Equivalent Concentration on Cells	10,000 µg/mL (10 mg/mL)	1000 µg/mL (1 mg/mL)	100 µg/mL (0.1 mg/mL)	10 µg/mL (0.01 mg/mL)	1 µg/mL (0.001 mg/mL)

NOTE: The amounts of test chemical weighed and Chemical Dilution Medium added may be modified from the amounts given above, provided that the targeted concentrations specified for each tier are tested.

Figure 1. Solubility Flow Chart**TIER 1**

STEP 1:	20 mg/mL test chemical (TC) in 0.5 mL Chemical Dilution Medium: <ul style="list-style-type: none"> • if TC soluble, then <u>STOP</u>. • if TC insoluble, then go to STEP 2.
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TIER 2

STEP 2:	2 mg/mL TC in medium – increase volume from STEP 1 by 10 (i.e., to 5 mL) <ul style="list-style-type: none"> • if TC soluble, then <u>STOP</u>. • if TC insoluble, then go to STEP 3.
STEP 3:	200 mg/mL TC in DMSO <ul style="list-style-type: none"> • if TC soluble, then <u>STOP</u>. • if TC insoluble, test at 200 mg/mL in ETOH. <ul style="list-style-type: none"> • if TC soluble, then <u>STOP</u>. • If TC insoluble, go to STEP 4.

TIER 3

STEP 4:	0.2 mg/mL TC in medium – increase volume from STEP 2 by 10 (i.e., to 50 mL) <ul style="list-style-type: none"> • if TC soluble, then <u>STOP</u>. • if TC insoluble, test at 20 mg/mL in DMSO – increase volume from STEP 3 by 10 (i.e., to 5 mL). <ul style="list-style-type: none"> • if TC soluble, then <u>STOP</u>. • if TC insoluble, test at 20 mg/mL in ETOH – increase volume from STEP 3 by 10 (i.e., to 5 mL). <ul style="list-style-type: none"> • if TC soluble, then <u>STOP</u>. • if TC insoluble, then go to STEP 5.
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TIER 4

STEP 5:	2 mg/mL TC in DMSO – increase volume from STEP 4 by 10 (i.e., to 50 mL) <ul style="list-style-type: none"> • if TC soluble, then <u>STOP</u>. • if TC insoluble, test at 2 mg/mL in ETOH – increase volume from STEP 4 by 10 (i.e., to 50 mL). <ul style="list-style-type: none"> • if TC soluble, then <u>STOP</u>. • if TC insoluble, then go to STEP 6.
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TIER 5

STEP 6:	0.2 mg/mL TC in 50 mL DMSO <ul style="list-style-type: none"> • if TC soluble, then <u>STOP</u>. • if TC insoluble, test at 0.2 mg/mL in 50 mL ETOH <ul style="list-style-type: none"> • <u>STOP</u>
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2. Mechanical Procedures

- a) The following hierarchy of mixing procedures will be followed to dissolve the test chemical:
 - 1) Add test chemical to solvent as in Tier 1 of **Table 2**.
 - 2) Gently mix. Vortex the tube (1 –2 minutes).
 - 3) If test chemical hasn't dissolved, use sonication for up to 5 minutes.
 - 4) If sonication doesn't work, then warm solution to 37°C. This can be performed by warming 5 mL tubes in a 37°C water bath for at least 5-10 minutes before evaluating solubility. Warm larger vessels for at least 15-20 minutes in a 37°C water bath before evaluating solubility.
 - 5) Proceed to Tier 2 (and Tiers 3-5, if necessary of Table 2 and repeat procedures 2-4).
- b) The preference of solvent for dissolving test chemicals is Chemical Dilution Medium, DMSO, and then ethanol. Thus, if (all solvents for a particular tier are tested simultaneously and) a test chemical dissolves in more than one solvent, then the choice of solvent follows this hierarchy. For example, if, at any tier, a chemical is soluble in Chemical Dilution Medium and DMSO, but not ethanol, the choice of solvent would be medium. If the chemical were insoluble in medium, but soluble in DMSO and ethanol, the choice of solvent would be DMSO.

After the lab has determined the preferred solvent for the test chemical and before proceeding to the cytotoxicity testing, the Study Director will discuss the solvent selection with the Study Management Team (SMT) of the validation study. The SMT will relate what solvent should be used in the assay for each chemical.

E. Preparation of Test Chemicals

[Note: Preparation under red or yellow light is recommended to preserve chemicals that degrade upon exposure to light.]

1. Test Chemicals in Solution

- a) Allow test chemicals to equilibrate to room temperature before dissolving and diluting.
- b) Prepare test chemical immediately prior to use. The solutions must not be cloudy nor have noticeable precipitate. Each stock dilution should have at least 1-2 mL total volume to ensure adequate solution for the test wells in a single 96-well plate.
- c) For chemicals dissolved in DMSO or ethanol, the final DMSO or ethanol concentration for application to the cells must be 0.5 % (v/v) in the vehicle controls and in all of the eight test concentrations.
- d) The stock solution for each test chemical should be prepared at the highest concentration found to be soluble in the solubility test. Thus, the highest test concentration applied to the cells in each range finding experiment is:

- 0.5 times the highest concentration found to be soluble in the solubility test, if the chemical was soluble in Chemical Dilution Medium, or
 - 1/200 the highest concentration found to be soluble in the solubility test if the chemical was soluble in ethanol or DMSO.
- e) The seven lower concentrations in the range finding experiment would then be prepared by successive dilutions that decrease by one log unit each. The following example illustrates the preparation of test chemical in solvent and the dilution of dissolved test chemical in Chemical Dilution Medium before application to 3T3 cells.

Example: Preparation of Test Chemical in Solvent Using a Log Dilution Scheme

If DMSO was determined to be the preferred solvent at Tier 2 of the solubility test (i.e., 200,000 µg/mL), dissolve the chemical in DMSO at 200,000 µg/mL for the chemical stock solution.

- 1) Label eight tubes 1 – 8. Add 0.9 mL solvent (e.g., DMSO) to tubes 2 -- 8.
- 2) Prepare stock solution of 200,000 µg test chemical/mL solvent in tube # 1.
- 3) Add 0.1 mL of 200,000 µg/mL dilution from tube #1 to tube #2 to make a 1:10 dilution in solvent (i.e., 20,000 µg/mL).
- 4) Add 0.1 mL of 20,000 µg/mL dilution from tube #2 to tube #3 to make another 1:10 dilution (i.e., 1:100 dilution from stock solution) in solvent (i.e., 2,000 µg/mL)
- 5) Continuing making serial 1:10 dilutions in the prepared solvent tubes.
- 6) Since each concentration is 200 fold greater than the concentration to be tested, make a 1:100 dilution by diluting 1 part dissolved chemical in each tube with 99 parts of Chemical Dilution Medium (e.g., 0.1 mL test chemical in DMSO + 9.9 mL Chemical Dilution Medium) to derive the eight 2X concentrations for application to 3T3 cells. Each 2X test chemical concentration will then contain 1 % v/v solvent. The 3T3 cells will have 0.05 mL Routine Culture Medium in the wells prior to application of the test chemical. By adding 0.05 mL of the appropriate 2X test chemical concentration to the appropriate wells, the test chemical will be diluted appropriately (e.g., highest concentration in well will be 1,000 µg/mL) in a total of 0.1 mL and the solvent concentration in the wells will be 0.5% v/v.
- 7) A test article prepared in Chemical Dilution Medium, DMSO, or ethanol may precipitate upon transfer into the Routine Culture Medium. The 2X dosing solutions should be evaluated for precipitates and the results will be recorded in the workbook. It will be permissible to test all of the dosing solutions in the dose range finding assay only. Doses containing test article precipitates should be avoided, and will not be used in the IC_x determinations for either the range finding experiments or the definitive tests.

Document all test chemical preparations in the Study Workbook.

2. pH of Test Chemical Solutions

Measure the pH of the highest concentration of the test chemical in culture medium using pH paper. Document the pH and note the color of the medium for all dilutions. Do not adjust the pH.

3. Concentrations of Test Chemical

a) Range Finder Experiment

Test eight concentrations of the test chemical by diluting the stock solution with a constant factor covering a large range. The initial dilution series shall be log dilutions (e.g., 1:10, 1:100, 1:1000, etc.).

The data from any well that has precipitate will be excluded from any calculations.

b) Main Experiment

[Note: After the range finding assay is completed, the concentration-response experiment shall be performed three times on three different days for each chemical (i.e., one plate per day per chemical).]

Depending on the slope of the concentration-response curve estimated from the range finder, the dilution/progression factor in the concentration series of the main experiment should be smaller ($\sqrt[6]{10} = 1.47$). Cover the relevant concentration range ($\geq 10\%$ and $\leq 90\%$ effect) preferably with three points of a graded effect, but with a minimum of two points, one on each side of the IC_{50} value, avoiding too many non-cytotoxic and/or 100%-cytotoxic concentrations. Experiments revealing less than one cytotoxic concentration on each side of the IC_{50} value shall be repeated, where possible, with a smaller dilution factor. In addition, the dilution scheme shall be adjusted in subsequent replicate assays (i.e., definitive assays), if necessary, to increase the number of points on both sides of the IC_{50} in the 10-90% response range. (Taking into account pipetting errors, a progression factor of 1.21 is regarded the smallest factor achievable.)

Determine which test chemical concentration is closest to the IC_{50} value (e.g., 50 % cytotoxicity). Use that value as a central concentration and adjust dilutions higher and lower in equal steps for the definitive assay.

Maximum Doses to be Tested in the Main Experiments

If minimal or no cytotoxicity was measured in the dose range finding assay, a maximum dose for the main experiments will be established as follows:

- For test chemicals prepared in Chemical Dilution Medium, the highest test article concentration that may be applied to the cells in the main experiments will be either 100 mg/mL, or the maximum soluble dose. Test chemical will be weighed into a glass tube and the weight will be documented. A volume of Chemical Dilution Medium will be added to the vessel so that the concentration is 200,000 $\mu\text{g/mL}$ (200 mg/mL). The solution is mixed as specified in **Section VII.D.2.a**. If complete solubility is achieved in medium, then 7 additional serial stock dosing solutions may be prepared from the 200 mg/mL 2X stock. If the test chemical is insoluble in medium at 200 mg/mL, proceed by adding medium, in small incremental amounts, to attempt to dissolve the chemical by using the sequence of mixing procedures specified in **Section VII.D.2.a**. The highest soluble stock solution will be used to prepare the 7 additional serial stock dosing solutions.
- For test chemicals prepared in either DMSO or ethanol, the highest test article concentration that may be applied to the cells in the main experiments will be either 2.5 mg/mL, or less, depending upon the maximum solubility in solvent. Test

chemical will be weighed into a glass tube and the weight will be documented. A volume of the appropriate solvent (determined from the original solubility test) will be added to the vessel so that the concentration is 500,000 µg/mL (500 mg/mL). The solution is mixed as specified in **Section VII.D.2.a**. If complete solubility is achieved in the solvent, then 7 additional serial stock dosing solutions may be prepared from the 500 mg/mL 200X stock. If the test chemical is insoluble in solvent at 500 mg/mL, proceed by adding solvent, in small incremental amounts, to attempt to dissolve the chemical by using the sequence of mixing procedures specified in **Section VII.D.2.a**. The highest soluble stock solution will be used to prepare the 7 additional serial stock dosing solutions.

c) Test Chemical Dilutions

The dosing factor of 3.16 ($= \sqrt[2]{10}$) divides a log into two equidistant steps, a factor of 2.15 ($= \sqrt[3]{10}$) divides a decade into three steps. The factor of 1.47 ($= \sqrt[6]{10}$) divides a log into six equidistant steps, and the factor of 1.21 ($= \sqrt[12]{10}$) divides the log into 12 steps.

EXAMPLE:

10						31.6						100
10				21.5				46.4				100
10		14.7		21.5		31.6		46.4		68.1		100
10	12.1	14.7	17.8	21.5	26.1	31.6	38.3	46.4	56.2	68.1	82.5	100

The technical production of decimal geometric concentration series is simple. An example is given for factor 1.47:

Dilute 1 volume of the highest concentration by adding 0.47 volumes of diluent. After equilibration, dilute 1 volume of this solution by adding 0.47 volumes of diluent...(etc.).

F. Test Procedure

1. 96-Well Plate Configuration

The 3T3 NRU assay for test chemicals will use the 96-well plate configuration shown in **Figure 2**.

Figure 2. 96-Well Plate Configuration for Positive Control and Test Chemical Assays

	1	2	3	4	5	6	7	8	9	10	11	12
A	b	b	b	b	b	b	b	b	b	b	b	b
B	b	VC	C ₁	C ₂	C ₃	C ₄	C ₅	C ₆	C ₇	C ₈	VC	b
C	b	VC	C ₁	C ₂	C ₃	C ₄	C ₅	C ₆	C ₇	C ₈	VC	b
D	b	VC	C ₁	C ₂	C ₃	C ₄	C ₅	C ₆	C ₇	C ₈	VC	b
E	b	VC	C ₁	C ₂	C ₃	C ₄	C ₅	C ₆	C ₇	C ₈	VC	b
F	b	VC	C ₁	C ₂	C ₃	C ₄	C ₅	C ₆	C ₇	C ₈	VC	b
G	b	VC	C ₁	C ₂	C ₃	C ₄	C ₅	C ₆	C ₇	C ₈	VC	b
H	b	b	b	b	b	b	b	b	b	b	b	b

VC = untreated VEHICLE CONTROL (mean viability set to 100 %)
 C₁ – C₈ = Test Chemicals or Positive Control (SLS) at eight concentrations
 (C₁ = highest, C₈ = lowest)
 b = BLANKS (contain **no** cells)

2. Application of Test Chemical

- Two optional methods for rapidly applying the 2X dosing solutions onto the 96-well plates may be utilized. The first method is to add each of the 2X dosing solutions into labeled, sterile reservoirs (e.g., Corning/Costar model 4870 sterile polystyrene 50 mL reagent reservoirs and/or Corning/Transtar model 4878 disposable reservoir liners, 8-channel). The second method utilizes a “dummy” plate (i.e., an empty sterile 96-well plate) prepared to hold the dosing solutions immediately prior to treatment of the test plate (with cells). The test chemical and control dosing solutions should be dispensed into the dummy plate in the same pattern/order as will be applied to the plate containing cells. More volume than needed for the test plate (i.e. greater than 50 µl/well) should be in the wells of the dummy plate. At the time of treatment initiation, a multi-channel micropipettor is used to transfer the 2X dosing solutions, from the reservoirs or dummy plate, to the appropriate wells on the treatment plate (as described in step c. below). These methods will ensure that the dosing solutions can be transferred rapidly to the appropriate wells of the test plate to initiate treatment times and to minimize the range of treatment initiation times across a large number of treatment plates, and to prevent “out of order” dosing.
- After 24 h ± 1 h incubation of the cells, remove Routine Culture Medium from the cells by careful inversion of the plate (i.e., “dump”) over an appropriate receptacle. Gently blot the plate on a sterile paper towel so that the monolayer is minimally disrupted. Do not use automatic plate washers for this procedure nor vacuum aspiration.
- Immediately add 50 µL of fresh pre-warmed Routine Culture Medium to all of the wells, including the blanks. Add 50 µL of Chemical Dilution Medium to the blank wells. Then add 50 µL Chemical Dilution Medium containing either the appropriate

concentration of test chemical, the PC, or the VC (see **Figure 2** for the plate configuration). The solutions will be transferred from the dummy plate to the test plate by adding the vehicle control first then lowest to highest dose so that the same pipette tips on the eight channel pipettor can be used for the whole plate.

- d) Incubate cells for $48 \text{ h} \pm 0.5 \text{ h}$ ($37^{\circ}\text{C} \pm 1^{\circ}\text{C}$, $90 \% \pm 5 \%$ humidity, and $5.0 \% \pm 1 \% \text{ CO}_2/\text{air}$).
- e) **Positive Control:** For each set of test chemical plates used in an assay, a separate plate of positive control concentrations will be set up following the concentration range established in the development of the positive control database in Phase Ia of the Validation Study. The mean IC_{50} and \pm two standard deviations (SD) of the IC_{50} of SLS (mutually agreed upon by the Testing Facility and the SMT) are the values that will be used as an acceptance criterion for test sensitivity for the 3T3 NRU assay. This plate will follow the same schedule and procedures as used for the test chemical plates.

3. Microscopic Evaluation

After at least 46 h treatment, examine each plate under a phase contrast microscope to identify systematic cell seeding errors and growth characteristics of control and treated cells. Record any changes in morphology of the cells due to the cytotoxic effects of the test chemical, but do not use these records for any quantitative measure of cytotoxicity. Undesirable growth characteristics of control cells may indicate experimental error and may be cause for rejection of the assay. Use the following Visual Observations Codes in the description of cell culture conditions.

Visual Observations Codes

Note Code	Note Text
1	Normal Cell Morphology
2	Low Level of Cell Toxicity
3	Moderate Level of Cell Toxicity
4	High level of Cell Toxicity
1P	Normal Cell Morphology with Precipitate
2P	Low Level of Cell Toxicity with Precipitate
3P	Moderate Level of Cell Toxicity with Precipitate
4P	High level of Cell Toxicity with Precipitate
5P	Unable to View Cells Due to Precipitate

4. Measurement of NRU

- a) Carefully remove (i.e., “dump”) the medium with test chemical and rinse the cells very carefully with 250 μL pre-warmed D-PBS. Remove the rinsing solution by dumping and remove excess by gently blotting on sterile paper towels. Add 250 μL NR medium (to all wells including the blanks) and incubate ($37^{\circ}\text{C} \pm 1^{\circ}\text{C}$, $90 \% \pm 5 \%$ humidity, and $5.0 \% \pm 1 \% \text{ CO}_2/\text{air}$) for $3 \pm 0.1 \text{ h}$. Observe the cells briefly during the NR incubation (e.g., at 1, 2, and 3 h – Study Director’s discretion) for NR crystal formation. Record observations in the Study Workbook. Study Director can decide to reject the experiment if excessive NR crystallization has occurred.

- b) After incubation, remove the NR medium, and carefully rinse cells with 250 μ l pre-warmed D-PBS.
- c) Decant and blot D-PBS from the plate.
- d) Add exactly 100 μ l NR Desorb (ETOH/acetic acid) solution to all wells, including blanks.
- e) Shake microtiter plate rapidly on a microtiter plate shaker for 20 – 45 min to extract NR from the cells and form a homogeneous solution.
- f) Measure the absorption (within 60 minutes of adding NR Desorb solution) of the resulting colored solution at 540 nm \pm 10 nm in a microtiter plate reader (spectrophotometer), using the blanks as a reference. [Phase Ia data show the mean OD value for the plate blanks to be 0.051 \pm 0.022 for 3T3 cells (\pm two standard deviations; data from 3 labs; N = 59). Use this value as a **guide** for assessment of the blank values.] Save raw data in the Excel format as provided by the SMT.

5. Quality Check of 3T3 NRU Assay

a) Test Acceptance Criteria

- 1) A test meets acceptance criteria, if the IC₅₀ for SLS (PC) is within \pm two (2) standard deviations of the historical mean established by the Test Facility (as per **VII.F.2.e**).
- 2) A test meets acceptance criteria if the corrected mean OD₅₄₀ of VCs is \geq 0.30 and \leq 0.80.
- 3) A test meets acceptance criteria if the left and the right mean of the VCs do not differ by more than 15 % from the mean of all VCs.
- 4) A test meets acceptance criteria if a minimum of two points, one on each side of the IC₅₀ value, are determined and fall within the range \geq 10 % and \leq 90 % effect.

[Note: All acceptance criteria must be met for an assay to be considered acceptable.]

b) Checks for Systematic Cell Seeding Errors

To check for systematic cell seeding errors, untreated VCs are placed both at the left side (row 2) and the right side (row 11 for the test plates) of the 96-well plate. Aberrations in the cell monolayer for the VCs may reflect a volatile and toxic test article present in the assay.

Checks for cell seeding errors may also be performed by examining each plate under a phase contrast microscope to assure that cell quantity is consistent.

c) Quality Check of Concentration-Response

The IC₅₀ derived from the concentration-response of the test chemicals will be backed by preferably three responses \geq 10 % and \leq 90 % inhibition of NRU and at least two responses, one on either side of the IC₅₀ value (see **VII.E.3.b**). If this is not the case, and the concentration progression factor can be easily reduced, reject the experiment and repeat it with a smaller progression factor. In addition, the dilution scheme shall be adjusted in subsequent replicate assays, if necessary, to increase the number of points on

both sides of the IC_{50} in the 10-90% response range. Numerical scoring of the cells (see **VII.F.3**) should be determined and documented in the Study Workbook.

G. Data Analysis

A calculation of cell viability expressed as NRU is made for each concentration of the test chemical by using the mean NRU of the six replicate values (minimum of four acceptable replicate well) per test concentration (blanks will be subtracted). This value is compared with the mean NRU of all VC values (provided VC values have met the VC acceptance criteria). Relative cell viability is then expressed as percent of untreated VC. If achievable, the eight concentrations of each chemical tested will span the range of no effect up to total inhibition of cell viability. Data from the microtiter plate reader shall be transferred to the Excel® spreadsheet provided by the SMT for determining cell viability and performing statistical analyses.

The concentration of a test chemical reflecting a 20 %, 50 %, and 80 % inhibition of cell viability (i.e., the IC_{20} , IC_{50} , and IC_{80}) is determined from the concentration-response by applying a Hill function to the concentration-response data. Statistical software (e.g., GraphPad PRISM® 3.0) specified by the SMT shall be used to calculate IC_{20} , IC_{50} , and IC_{80} values (and the associated confidence limits) for each test chemical. In addition, the SMT shall provide guidelines for calculating IC_x values and confidence limits. The Testing Facility shall report data using at least three (3) significant figures and shall forward the results from each assay to the SMT/biostatistician through the designated contacts in electronic format and hard copy upon completion of testing. The SMT will be directly responsible for the statistical analyses of the Validation Study data.

VIII. REFERENCES

Hackenberg, U. and H. Bartling. 1959. Messen und Rechnen im pharmakologischen Laboratorium mit einem speziellen Zahlensystem (WL24-System). Arch. Exp. Pathol. Pharmacol. 235: 437-463.

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IX. APPROVAL

SPONSOR REPRESENTATIVE
(Print or type name)

DATE

Test Facility STUDY DIRECTOR
(Print or type name)

DATE